

# 19,19'-Diacyloxy Signature: An Atypical Level of Structural Evolution in Carotenoid Pigments

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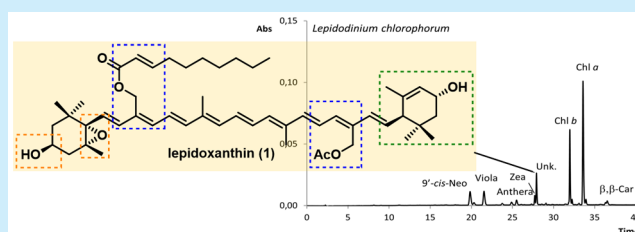
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## S Supporting Information

**ABSTRACT:** We report the isolation from the green dinoflagellate *Lepidodinium chlorophorum* and structural characterization of a new carotenoid termed lepidoxanthin (1), determined to be (3S,5R,6S,3'R,6'R)-5,6-epoxy-19-(2-decenoyloxy)-19'-acetoxy-4',5'-didehydro-5,6,5',6'-tetrahydro- $\beta,\epsilon$ -carotene-3,3'-diol. Its until now unidentified 19,19'-diacyloxy substitution constitutes a chemical signature that can aid in unraveling the evolutionary course of this unicellular algae based on the proposed biosynthetic pathway.



The endosymbiotic theory accredits the origin of photosynthetic eukaryote evolution in a first event of endosymbiosis when a host eukaryotic cell ingested an ancestral cyanobacterium. Through this first association three lineages of algae were differentiated: Glaucophyta, Rhodophyta (red algae), and Chlorophyta (green algae). Successive events of endosymbiosis led to the present diversity of algal groups (Figure 1).

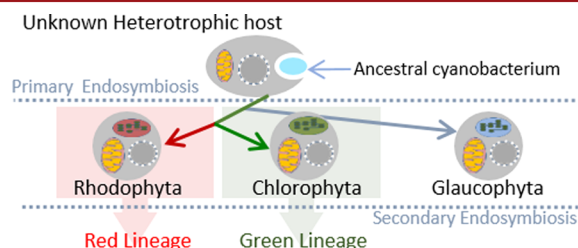


Figure 1. Schematic representation of algal plastid evolution.

In this process, the complexity and internal organization of these photosynthetic eukaryotes have concurrently increased through the partial transfer of the engulfed cyanobacteria genes to the host nucleus. An ultimate evidence of this evolution is the more complex algal carotenoids produced by more sophisticated biosynthetic pathways. Consequently, both genes<sup>1</sup> and enzymes involved in carotenogenesis, as well as the photosynthetic pigments produced, provide hints to construct the evolution picture.<sup>2,3</sup> In addition, some carotenoids are found only in specific algal divisions or classes so they can be used as chemotaxonomic markers.<sup>4</sup>

A case study is the green dinoflagellate genus *Lepidodinium*, which occupies a totally singular place in the evolution of

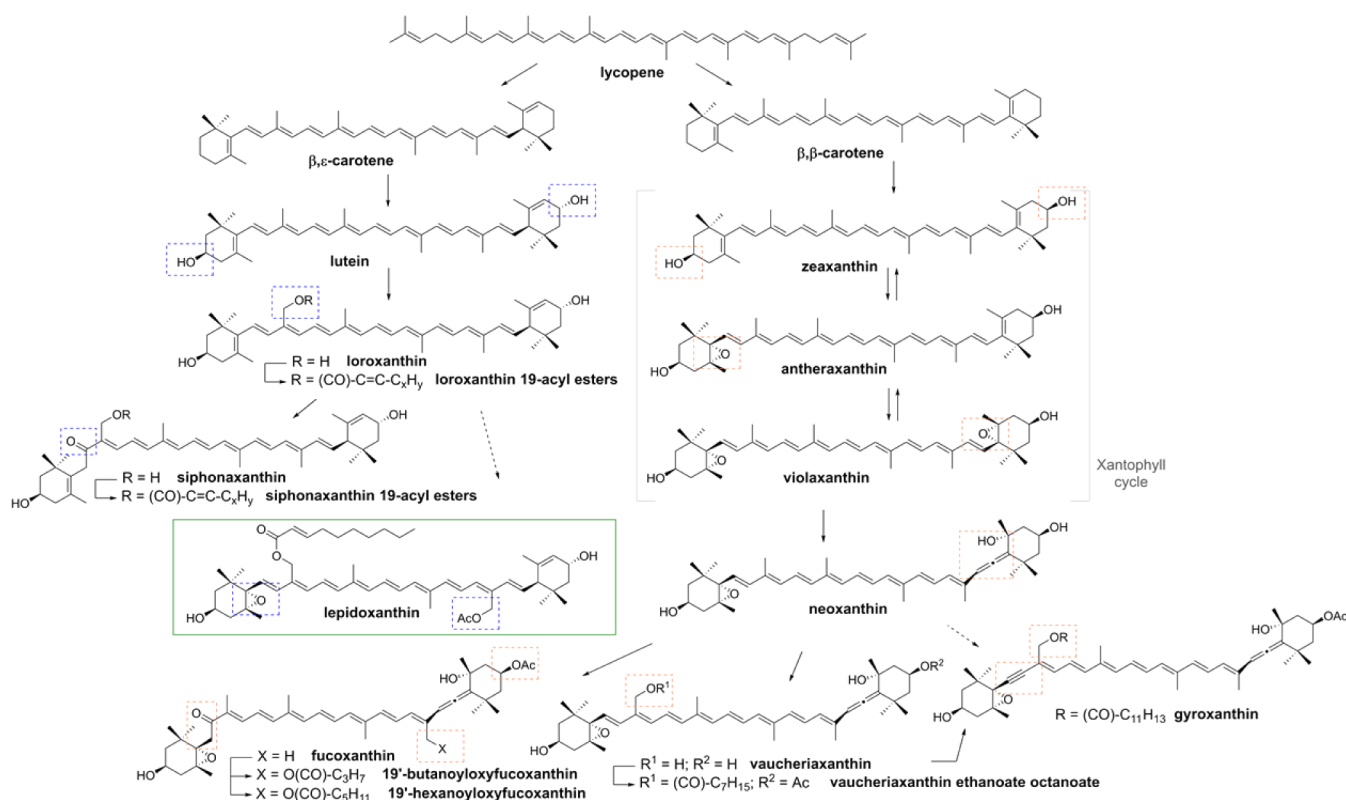
photosynthetic organisms: originated from the red evolutive lineage but featuring chloroplasts typical from the green algae (Figure S1). Only two species populate this particular genus: *L. chlorophorum* and *L. viride*, and both species can build up extensive and eventually harmful blooms in temperate coastal areas.<sup>5,6</sup> From the point of view of pigment composition, a common pigment pattern in core chlorophytes was previously assigned, with 9'-cis-neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, and lutein as main carotenoids.<sup>7,8</sup> These xanthophylls are mainly associated with photosynthetic and photoprotective functions in algae.<sup>9</sup> However, we subsequently found spectral<sup>10</sup> and chromatographic<sup>11</sup> evidence indicating that *L. chlorophorum* deviates from this general composition, featuring an unknown xanthophyll with similar chromatographic properties instead of lutein as the main carotenoid. Given the fact that the different molecular structures of carotenoids related to specific biosynthesis pathways can aid in unraveling the evolutive course of photosynthetic organisms, we decided to isolate (see Supporting Information) this unknown pigment and unequivocally determine its structure by analysis of UV, MS, and NMR data.

Our results suggest a novel xanthophyll structure with a double in-chain acyl substitution in central methyl groups. 19- or 19' acyloxy-substituted carotenoids occur along many algal taxons, biosynthetically produced by the two carotenogenesis pathways derived from lycopene (Figure 2).

Belonging to the  $\beta,\epsilon$ -carotene biosynthesis pathway,  $\alpha,\beta$ -unsaturated acyl esters of siphonaxanthin and loroxanthin were identified in green algae.<sup>12</sup> Alternatively, the  $\beta,\beta$ -carotene

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**Figure 2.** Biosynthetic pathway of carotenoids from lycopene to in-chain hydroxylated carotenoids through  $\beta,\epsilon$ -carotene and  $\beta,\beta$ -carotene. To this class belong siphonaxanthin, loroxanthin, 19'-alkoxyfucoxanthins, vaucheriaxanthin, and gyroxanthin. Changes in each molecule are highlighted.

biosynthetic route that comprises the photoprotective xanthophyll cycle (zeaxanthin, antheraxanthin, and violaxanthin) as the central pigment provided 19-acylated carotenoids as 19'-alkoxyfucoxanthins, gyroxanthin, and lipidated vaucheriaxanthin, identified in the red evolutive lineage.<sup>4,13</sup>

We initiated this study comparing the pigment composition of *L. chlorophorum* and the pedinophyte *Pedinomonas minor*, reported to be highly related by previous gene analysis.<sup>14</sup> HPLC–PDA vis spectroscopy confirmed that although both peaks coelute (27.6 min) in HPLC,<sup>15</sup> which would explain the misidentification by Matsumoto et al.,<sup>8</sup> the spectra of lutein in *P. minor* and the unknown carotenoid from *L. chlorophorum* show discrepancies in both maxima and fine structure (Figure S2).<sup>16</sup> The sample of the unknown pigment isolated from the culture of *L. chlorophorum* was incubated in dilute hydrochloric acid to test the presence of 5,6-epoxides. A characteristic hypsochromic shift of the UV/vis spectrum of 20 nm evidenced the 5,6-epoxide rearrangement into a 5,8-analogue and the subsequent shortening of the chromophore, therefore giving a positive outcome.<sup>17</sup>

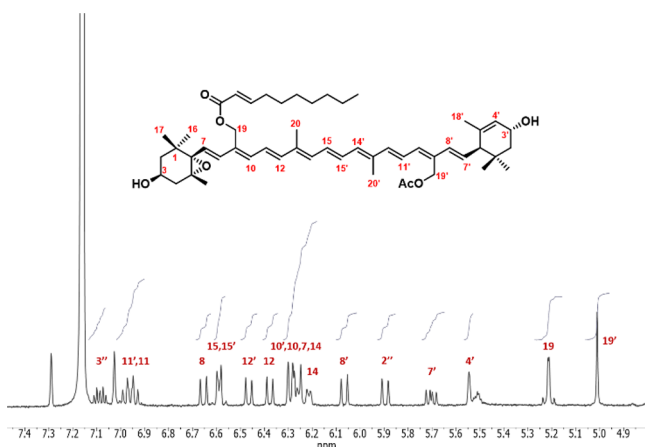
Analysis of the ESI high-resolution mass spectrum showed  $[M + Na]^+$  and  $[M + H]^+$  peaks (at  $m/z$  833.5298 and 811.5491, respectively) compatible with the neutral formula  $C_{52}H_{74}O_7$ , featuring a difference of 12 carbon atoms with respect to the basic  $C_{40}$ -carotenoid skeleton. An important peak at  $m/z$  641.4194 ( $C_{42}H_{57}O_5$ ,  $[M + H - C_{10}H_{18}O_2]^+$ ) was also detected, which could be assigned to the in-source loss of decenoic acid (–170 Da). The analysis of the MS/MS spectra showed abundant ions ( $m/z$  773.5097,  $[M + Na - 60]^+$  and  $m/z$  581.3975,  $[M + H - 170 - 60]^+$ ) due to the neutral loss of 60 Da ( $C_2H_4O_2$ , acetic acid) characteristic of an acetylated structure. Further accumulation of neutral losses of 18 Da pointed to the presence of two additional hydroxyl groups in the periphery of the carotenoid skeleton.

Additionally, a peak was observed at  $m/z$  181.1221, a fragment characteristic of 3-hydroxylated carotenoid epoxides ( $C_{11}H_{17}O_2$ ), attributed to the fission of C8–C9 bond and formation of the corresponding furanoid oxocarbenium ion.<sup>18</sup>

Further chemical analysis was made by saponification of the sample, showing an important shift in the HPLC chromatogram to lower retention time (7.5 min) while preserving a completely similar UV/vis spectrum. High-resolution ESI analysis of this sample provided an abundant  $[M + H]^+$  peak (at  $m/z$  617.4193) in accordance with the neutral formula  $C_{40}H_{56}O_5$ , which correlates well with the free carotenoid after elimination of acetic and decenoic acids. Also similarly, important ion peaks were observed from successive 18 Da differences, compatible with the presence of four hydroxyl groups in the de-esterified pigment.

Detailed structural information was obtained from 1D- and 2D-NMR spectroscopy. In order to avoid degradation of the sample during acquisition, benzene- $d_6$  was selected as a suitable solvent to perform the structural elucidation. The  $^1H$  NMR spectrum (see Supporting Information) showed 17 differentiated olefinic protons in the low-field region, denoting an unsymmetrical carotenoid skeleton (Figure 3). Notably, two additional signals in the proximal  $\delta$  5.5–5.0 ppm region integrating for two protons, respectively, were observed: (a) an AB system at  $\delta$  5.23 ppm and  $\delta$  5.20 ppm, resembling the oxymethylene group of the 19'-acyloxy substitution of vaucheriaxanthin,<sup>19,20</sup> and (b) a singlet at slightly higher field, attributable to an analogous moiety.

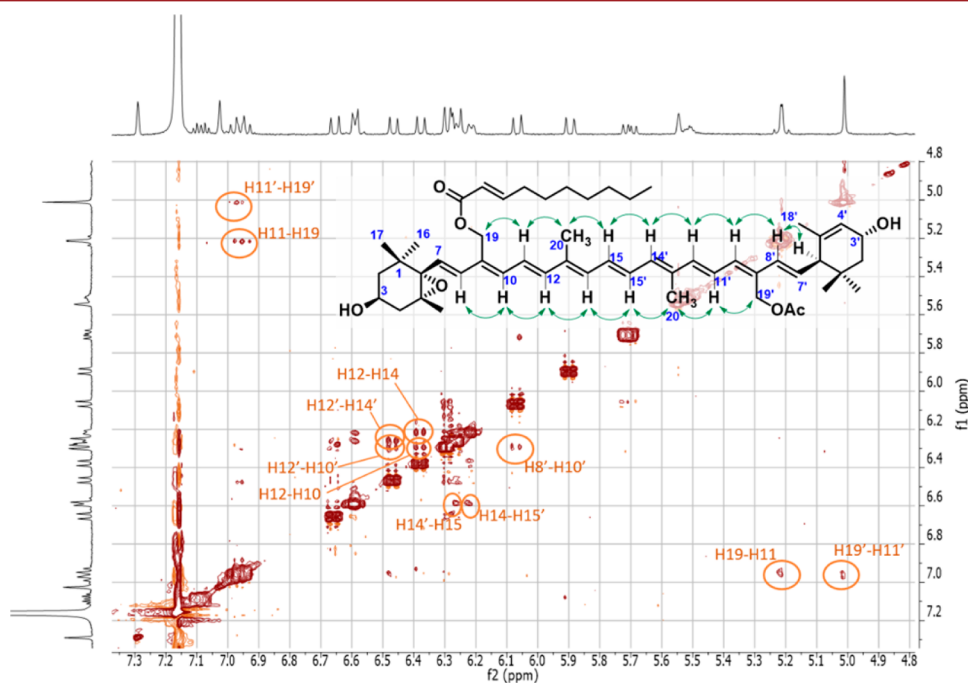
In previously reported 19- or 19'-acyloxy-substituted carotenoids, the in-chain acyl substitution in a methyl group is observed as an AB system in *all-trans* polyenic surroundings, probably due to a rotational restriction of the C9–C19 bond or



**Figure 3.** Low-field region of the  $^1\text{H}$  NMR spectrum of the isolated pigment and proposed structure for lepidoxanthin (1).

as a singlet in acetylenic structures that are less sterically blocked.<sup>21</sup> In our case, the bulkiness of the ester substituents should be markedly different, as the AB system is induced by the bulkier ester and the singlet is influenced by an ester of smaller size, compatible with a rapid rotation in the NMR time scale. This proposed novel 19,19'-diacylated central polyene structure is further supported by the spatial correlations identified in the NOESY spectra (Figure 4). Crucial are the observed correlations between these assigned oxymethylene protons with the proximal H11 and H11' (dd at  $\delta$  6.29 ppm and  $\delta$  6.38 ppm). It is important to note that both distribution and shape of this spectral region closely resemble the 19'-acyl structure of moraxanthin (a hexanylester of vaucheriaxanthin) previously characterized by 1D- and 2D-NMR (benzene- $d_6$ ).<sup>21</sup> As a result of the analogous nature of the oxygenated substituents at the central chromophore (at 19 and 19' positions), a high symmetry was expected for the opposite polyenic spin systems, being differentiated

almost exclusively by the corresponding terminal groups. In order to confirm this hypothesis, a careful analysis of the coupled spin systems was performed. The COSY spectrum showed two equivalent spin–spin coupling correlations between H10,11,12 and H10',11',12' protons. Thus, two double doublets compatible with H11 and H11' (at  $\delta$  6.95 ppm and  $\delta$  6.97 ppm, respectively) showed strong individual cross-peaks with two additional doublets: the vicinal H10 and H12 vinyl protons for H11 (at  $\delta$  6.29 ppm and  $\delta$  6.38 ppm) and H10', H12' (at  $\delta$  6.29 ppm and  $\delta$  6.47 ppm) for H11'. The central polyene system was assigned to the coincident H15–H15' and the two closely positioned doublets of H14 and H14' (at  $\delta$  6.21 ppm and  $\delta$  6.26 ppm). A markedly deshielded chemical shift (dt at  $\delta$  7.09 ppm) was easily observed, pointing out the presence of an  $\alpha,\beta$ -unsaturated ester, which together with the doublet at  $\delta$  5.90 ppm could be ascribed to a 2-decenoyloxy group at C19. Additionally, an AX system at  $\delta$  6.65 ppm (H8) and  $\delta$  6.26 ppm (H7) clearly affected by the presence of an electron-withdrawing group was detected, compatible with a 5,6-epoxide- $\beta$ -ring. Supporting also the presence of a 3-hydroxy-5,6-epoxide- $\beta$ -ring, a multiplet at  $\delta$  3.82–3.75 ppm, which is consistent with the proton on C3 being adjacent to the hydroxyl group, was identified. Other important correlations among the protons of the ring were observed, including H3–H2 $\alpha,\beta$ , H3–H4 $\alpha,\beta$ , H2 $\alpha$ –H2 $\beta$ , and H4 $\alpha$ –H4 $\beta$  as well as cross-link correlations between H2 and H4 protons. Another spin system formed by three differentiated protons was also identified, consisting of two doublets at  $\delta$  6.07 ppm (H8',  $J$  = 15.6 Hz) and  $\delta$  2.32 ppm (H6',  $J$  = 9.9 Hz) and a doublet of doublets at  $\delta$  5.70 ppm (H7',  $J$  = 15.6 and 9.9 Hz), which is consistent with an  $\epsilon$ -ring (common in natural carotenoids such as lutein). In addition, the 2D-spectrum also supports the structure of the 3-hydroxy- $\epsilon$ -ring, with cross peaks between a vinyl proton at  $\delta$  5.55 ppm and a 3'-OH vicinal proton at  $\delta$  4.15–4.10 ppm. Further correlations such as H2' $\alpha$ –H2' $\beta$ , H2' protons with H3',



**Figure 4.** NOESY spectrum of the novel carotenoid isolated from *L. chlorophorum* (in  $\text{C}_6\text{D}_6$ ). The spatial correlations observed are represented in the structure proposed.



H3' with H4' and H3' with H18', and H4' with H18' were observed.

In addition to all of the vicinal coupling, the COSY spectrum also revealed distal proton–proton couplings, which aided in the assignment of the methyl groups on the polyene system by the correlations with olefinic protons. That was the case for H20, H20', and H18'. Furthermore, NOESY spectrum was essential to demonstrate the *all-trans* geometry of the double bonds, displaying cross-peaks of H8–H10, H11–H20, H8'–H10', and H11'–H20', which confirmed the *trans* configuration of the trisubstituted alkenes in the polyene chain. The absolute and relative configuration of the stereogenic centers of both terminal groups was proposed on the basis of the structures of well-defined naturally occurring carotenoids<sup>22</sup> and the delineated biosynthetic origin from lutein. Thus, both the  $\beta$ - and  $\epsilon$ -ring exhibit a *trans* relationship between the hydroxyl group at C3 and the substituents at C6. The resonance positions of the protons from the  $\beta$ -ring and the  $\epsilon$ -ring previously reported are in good agreement with the data observed for the newly isolated xanthophyll.

In conclusion, a new class of carotenoids was identified with a marine origin, featuring a doubly oxygenated central chromophore, as the first time that a double in-chain acyloxy group substitution is described. The proposed structure was designated as lepidoxanthin (**1**) in regard to its origin (*L. chlorophorum*), and was established as (3S,5R,6S,3'R,6'R)-5,6-epoxy-19-(2-decenoyloxy)-19'-acetoxo-4',5'-didehydro-5,6,5',6'-tetrahydro- $\beta,\epsilon$ -carotene-3,3'-diol. To date, such a structure has only been detected in the genus *Lepidodinium*, thus proving this carotenoid's important role as biomarker in chemotaxonomical and ecological (oceanographic) studies.

From an evolutionary point of view, the finding of this carotenoid in *Lepidodinium* establishes the origin of its plastids in classes of green algae with important oxidative capability along carotenogenesis, being able to acylate central positions of carotenoids. In this category, siphonaxanthin and loroxanthin are characteristic xanthophylls of certain classes of these green algae. In the case of *Lepidodinium*, this oxidizing capability is markedly higher, not showing lutein or other  $\beta,\epsilon$ -carotene-derived carotenoids but exclusively the highly oxidized lepidoxanthin (**1**). The structural function of this lipidated carotenoid may be related to a fine-tuned location in the light-harvesting complex,<sup>23</sup> where the steric hindrance of the long ester group could force a delineated location to efficiently absorb light energy.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02272.

Experimental details of culture, isolation and chemical tests; NMR, UV–vis and ESI-MS spectra of **1** (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ DEDICATION

We dedicate this work to Prof. Angel R. de Lera (Departamento de Química Orgánica, Universidade de Vigo, Spain) on his 60th birthday for a fruitful career with important contributions on the development of new strategies directed to the stereocontrolled synthesis of carotenoids.

## ■ REFERENCES

- (1) Tirichine, L.; Bowler, C. *Plant J.* **2011**, *66*, 45.
- (2) Schubert, N.; García-Mendoza, E. *J. Phycol.* **2006**, *42*, 1208.
- (3) Dorrell, R. G.; Howe, C. J. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 10247.
- (4) Takaichi, S. *Mar. Drugs* **2011**, *9*, 1101.
- (5) Reñé, A.; Satta, C. T.; Garcés, E.; Massana, R.; Zapata, M.; Anglès, S.; Camp, J. *Harmful Algae* **2011**, *12*, 11.
- (6) Morozov, E.; Pozdnyakov, D.; Smyth, T.; Sychev, V.; Grassl, H. *Int. J. Remote Sens.* **2013**, *34*, 1297.
- (7) Laza-Martinez, A.; Seoane, S.; Zapata, M.; Orive, E. *J. Plankton Res.* **2007**, *29*, 913.
- (8) Matsumoto, T.; Kawachi, M.; Miyashita, H.; Inagaki, Y. *J. Plant Res.* **2012**, *125*, 705.
- (9) Brunet, C.; Johnsen, G.; Lavaud, J.; Roy, S. In *Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography*; Roy, S., Llewellyn, C. A., Egeland, E. S., Johnsen, G., Eds.; Cambridge University Press: Cambridge, 2011; pp 445–471.
- (10) Zapata, M.; Fraga, S.; Rodríguez, F.; Garrido, J. *Mar. Ecol.: Prog. Ser.* **2012**, *465*, 33.
- (11) Sanz, N.; García-Blanco, A.; Gavalás-Olea, A.; Loures, P.; Garrido, J. L. *Methods Ecol. Evol.* **2015**, *6*, 1199.
- (12) Garrido, J. L.; Rodríguez, F.; Zapata, M. *J. Phycol.* **2009**, *45*, 366.
- (13) Jeffrey, S. W.; Wright, S. W.; Zapata, M. In *Phytoplankton Pigments: Characterization, Chemotaxonomy and Applications in Oceanography*; Roy, S., Llewellyn, C. A., Egeland, E. S., Johnsen, G., Eds.; Cambridge University Press: Cambridge, 2011; pp 3–77.
- (14) Kamikawa, R.; Tanifuji, G.; Kawachi, M.; Miyashita, H.; Hashimoto, T.; Inagaki, Y. *Genome Biol. Evol.* **2015**, *7*, 1133.
- (15) Zapata, M.; Rodríguez, F.; Garrido, J. L. *Mar. Ecol.: Prog. Ser.* **2000**, *195*, 29.
- (16) The hypsochromic shift observed for lepidoxanthin is compatible with a shorter conjugation system (from 10 insaturations in lutein to nine in lepidoxanthin). Additionally, the increased fine structure in comparison to the lutein spectrum points to a nonconjugated terminus ring at both ends for lepidoxanthin.
- (17) Britton, G. In *Carotenoids. Vol. 1B: Spectroscopy*; Britton, G., Liaaen-Jensen, S., Pfander, H., Eds.; Birkhäuser: Basel, 1995; pp 13–62.
- (18) Baldas, J.; Porter, Q. N. *Chem. Commun.* **1966**, 852.
- (19) Nitsche, H.; Egger, K. *Tetrahedron Lett.* **1970**, *11*, 1435.
- (20) Nitsche, H. *Z. Naturforsch.* **1973**, *28*, 641.
- (21) Mangoni, O.; Imperatore, C.; Tomas, C. R.; Costantino, V.; Saggiomo, V.; Mangoni, A. *Mar. Drugs* **2011**, *9*, 242.
- (22) Deli, J.; Ósz, E. *ARKIVOC* **2004**, 150.
- (23) Wang, W.; Qin, X.; Sang, M.; Chen, D.; Wang, K.; Lin, R.; Lu, C.; Shen, J.-R.; Kuang, T. *Photosynth. Res.* **2013**, *117*, 267.